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Evaluation of human corneal ulcer healing process using optical coherence tomography: an *in vitro* study

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ABSTRACT

Corneal injury is potentially leading to ulceration which remains a major health concern in ocular surface diseases. *In vitro* studies of new ophthalmic drugs selection are usually performed using excised cornea from slaughtered animals or laboratory animals. However, the outcomes from animal models do not completely reflect the human corneal repair and regeneration process. *In vitro* human corneal models suit better for the rapid testing of the drug uptake in response to drug's administration and posology. Therefore, this study aims at establishing an in-vitro 3D corneal model to characterise the corneal wound healing process. Moreover, a functional assessment of corneal morphology and strength change during the healing process is of urgent need. A phase-sensitive optical coherence tomography (OCT) system with a spectral-domain configuration was utilised to probe the structure and mechanical strength of the wounded corneal tissues. In this preliminary study, a human corneal 3D model was successfully established using tissue-engineering techniques and corneal injuries were mimicked with adjustable lesion size and depth. During the healing process, OCT provided an accurate indication of the tissue repair and regeneration. These results will be of great clinical impact to understand the biomechanics of the cornea healing process and the therapeutic effectiveness of regenerative medicine.

Keywords: Cornea, human keratocytes, *in vitro* model, wound healing, optical coherence tomography

1. INTRODUCTION

In vivo assessment of human corneal wound healing is problematic after injury and refractive surgery due to light absorption and scattering through the cornea. The cornea plays an essential role in focusing the vision. It is also a protective barrier against dirt, germs, and other potential damage that can harm the eye. However, the cornea is also exposed to damages, such as injuries, scratches, laser surgery and chemical reagents. The cornea usually heals on its own after minor injuries, but deeper injuries can cause corneal scarring, which will result in a haze on the cornea that impairs vision. Anti-inflammatory drugs and antibiotics have been used to reduce the infection and regulate the immune system to promote the wound healing process. *In vitro* studies of new ophthalmic drugs selection are usually performed using excised cornea from slaughtered animals or laboratory animals^{1,2}. However, animal models seems inappropriate³ due to the ethic concern, the short of animal models, as well as the inherent differences between human and animal eye in the aspects of structure and physiology. To avoid the use of animal experiments, reconstructed *in vitro* models⁴⁻⁷ using tissue engineering techniques proved to suit better for the rapid testing of the uptake or metabolism of drugs in corneal cells.

The tissues of the cornea are arranged in five different layers. Among them, the stroma contributes approximately 90% of the thickness of the entire cornea. In many connective tissues such as tendon and corneal stroma, collagen is the major mechanical and structural protein in the extracellular matrix (ECM), where collagen type I is the most abundant. The tissue-engineered corneal equivalents consist of collagen hydrogels seeded with corneal cells. Previous studies^{4,6,8-11} successfully reconstructed collagen based hydrogels for the application of collagen crosslinking treatments, monitoring cell and matrix interaction. Corneal keratocytes are specialised fibroblasts residing in the stroma. Fibroblasts was reported to remodel the surrounding ECM by applying force¹² and release ECM products¹³, thereby the mechanical strength can be modulated by fibroblasts seeded in the collagen hydrogel. Mechanical characterization of the corneal equivalents plays an essential role in studying the wound healing process and effects of new ophthalmic drugs as the pathological process is usually associated

with the mechanical strength of the cornea. A spherical indentation technique⁹⁻¹¹ has been used to measure the stiffness of the cornea model. However, it requires the hydrogel clamped during the measurement and a stainless steel ball is in contact with the hydrogel to perform deformation.

Optical coherence elastography (OCE) is a novel and non-invasive optical imaging technique aiming at early detection of subtle stiffness changes in diseased tissues. Vibration OCE stands out from different OCE methods with high resolution and large dynamic range. For detection of the tissue response, the deformation or displacement in tissue is measured using optical coherence tomography (OCT), and then the tissue stiffness or elasticity is resolved. The details of the theory has been described in our previous work¹⁴. This technique has been applied in a variety of tissues of small scale¹⁵ and in the detection of prostate cancer^{16,17}. This project aims at establishing an 3D corneal model *in vitro* and creating lesions with adjustable lesion size. In the study of corneal wound healing process, OCT is introduced to realise real-time and non-contact measurement the morphology and stiffness alteration of an injured corneal model. The success of this project will be of great clinical significance to understand the cell behaviour *in vivo* after injury and the therapeutic effectiveness of regenerative medicine.

2. METHODOLOGY

2.1 Human primary keratocytes

Human primary keratocytes (P10872; Innoprot) were cultured in T25 cell culture flasks containing Dulbecco's-modified Eagle medium (DMEM w/1.0 g/L glucose w/o L-Glutamine; Lonza) supplemented with Foetal Bovine Serum (FBS; 10% [v/v]; Gibco), antibiotic and antimetabolic solution (1% [v/v]; PEN-STREP 5000 U Penicillin/m, 5000U Streptomycin/ml; Lonza) and L-Glutamine (2 mM; Lonza), namely fibroblast medium at 37°C, 5% CO₂. The medium was changed every two days until 95% confluency. The keratocytes were then trypsinized to the next generation.

2.2 *In vitro* corneal model and lesion

The *in-vitro* corneal model was developed using P3 keratocyte/fibroblasts and type I collagen as illustrated in Figure 1. Collagen I (Rat tail, Corning®) was mixed with 10X DMEN, 1N NaOH and cell suspension. All ingredients were kept on ice during this procedure to prevent premature gelation. Solution was mixed by pipetting the mixture upwards and downwards, ensuring that no air bubbles were made. The required volume for each component was calculated following the instruction with the product. However, 10X PBS in the original instruction was substituted with 10X DMEN, and sterile dH₂O was substituted with cell suspension. A final collagen concentration of 3.2 mg/mL was applied throughout.

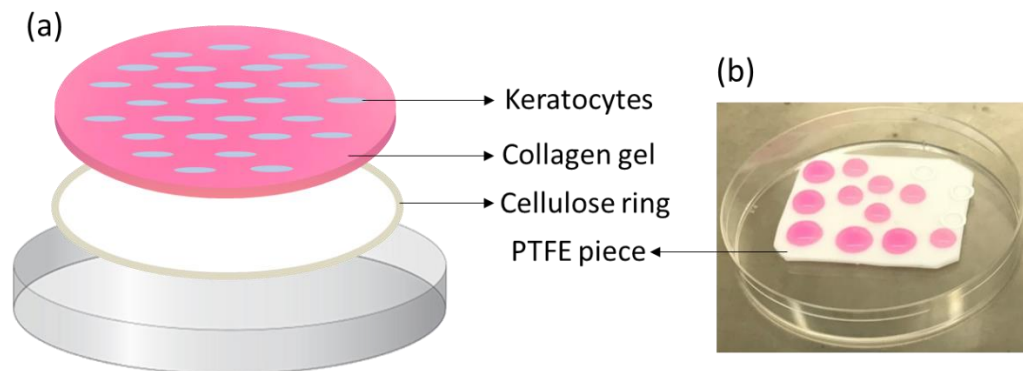


Figure 1 (a) A sketch of the design of *in-vitro* 3D corneal model and (b) the established stromal equivalent in petri dish.

Once mixed, the solution was carefully pipetted onto a cellulose ring of 7 mm outer diameter and 6 mm inner diameter placed on polytetrafluoroethylene (PTFE) pieces. Such rings were prepared to prevent the horizontal contraction of the gel.

After 2 hours, the mixture of cells and collagen became fully solidified. The collagen gel matrix seeded with keratocytes was then maintained for three days in the fibroblast medium at 37°C, 5% CO₂. The corneal lesion was mimicked using the biopsy punch of different size varying from 350 µm to 2 mm. For each size, five human miniature corneas were prepared for lesion mimicking. Moreover, a control group was also included where there were five hydrogels with lesion but without cells seeded.

2.3 Cell viability and morphology

Cell morphology was observed using light microscopy (Olympus CKX41) constantly. Cell viability was observed using a live-dead fluorescent double staining kit (Invitrogen™) according to the manufacturer's instructions. The culture plate was wrapped in aluminum foil to protect from light exposure and then incubated for 30 minutes at 37°C before imaging. Samples were then visualized using a confocal microscope (Olympus FluoView FV1200 Confocal Laser Scanning Microscope).

2.4 Optical Coherence Elastography (OCE)

The OCE system consists of two main parts: signal detection and vibration stimulation as illustrated in Figure 2. For signal detection, a phase-sensitive optical coherence tomography (PhS-OCT) with spectral-domain configuration was adapted to measure the sample displacement. The PhS-OCT system employs a superluminescent diode (SLD, Thorlabs) as a broadband light source, with a centre wavelength of ~1310 nm and bandwidth of ~83 nm. For the system performance, it provides an axial resolution of 8.9 µm and a transverse resolution of 18 µm in air. To generate vibration, a sine-wave signal modulated at ~8 kHz is generated by a function waveform generator (Agilent Technologies, USA) and an amplifier (AE Techtron). The signal is then sent to drive a magnet shaker (Brüel & Kjær Sound & Vibration Measurement A/S, Denmark). To form an M-scan image, the PhS-OCT beam stayed at one location of the sample for 512 repeats. A complete B-scan (a cross-section view) consists of 512 × 512 A-scans. Three-dimensional (3D) structure was then realized with consecutive B scans. Finally the raw structure and elastogram data sets were processed by MATLAB R2017b (The MathWorks, Natick, MA, USA) to generate structural and elastogram frames for each B-scan¹⁴. These frames were then imported into Amira (Mercury Computer Systems, Berlin, Germany) and reconstructed into 3D data sets at full resolution.

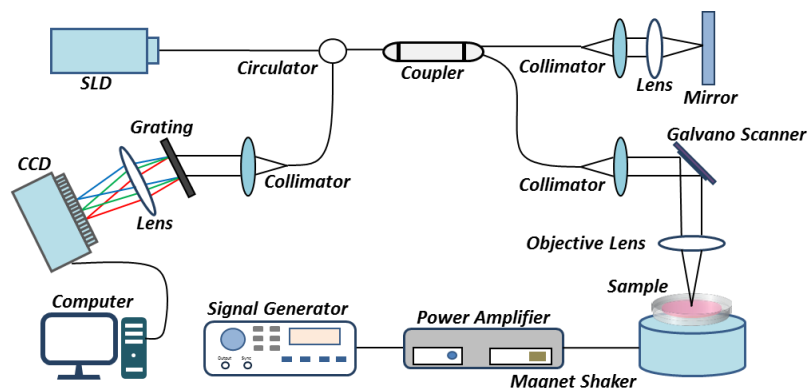


Figure 2. A schematic of system setup of the vibration OCE system for monitoring the corneal wound healing process. SLD (superluminescent diode), CCD (charged coupled device) line-scan camera.

Before OCT/OCE imaging, the culture medium was removed from each sample in the 48-well culture plate. The corneal models were then transferred to the 35-mm petri dish in the flow hood. To maintain a sterile environment inside the culture plate and continue culturing during the wound healing period, the samples needed to remain covered by a lid while imaging. The samples were protected and sealed using Parafilm to keep hydrated while imaging without causing reflection and image distortion. A sample holder was designed to attach to the shaker and fit the 35-mm petri dish in place, so the triggered

vibration was generated within the sample in the axial direction. While the shaker continued to fire the stimulus to the sample, the generated vibration signal was then acquired using M-B mode.

3. RESULTS

3.1 Keratocyte morphology

Resuscitated keratocytes in monolayer culture present a fibroblastic cell morphology as illustrated in Figure 3(a). Such attached cells have bipolar or multipolar characteristics and elongated shapes. The morphology change of the cells was recorded using the light microscopy every day. Typically, corneal keratocytes of 1.25×10^5 cells need 4 days to reach 95% confluency. After trypsinization, keratocytes were seeded in the 3D collagen matrix as shown in Figure 3(b). Figure 3(c) is a confocal image with higher contrast showing a stellate morphology of keratocytes.

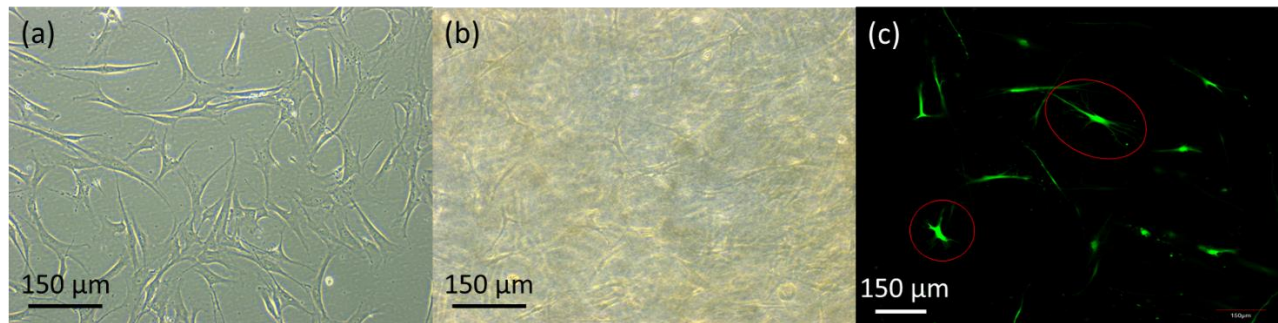


Figure 3. Keratocyte cell morphology in (a) 2D monolayer, (b) 3D collagen gel using light microscope and (c) confocal imaging after culturing for 2 days. Red circles illustrate keratocyte with stellate shape.

3.2 Cross-sectional view of corneal wound healing

Figure 4 demonstrates the cross-sectional OCT images of wound healing process during 2 weeks' culturing period of a corneal model with a 1-mm punched lesion. Images were taken from the same location every day.

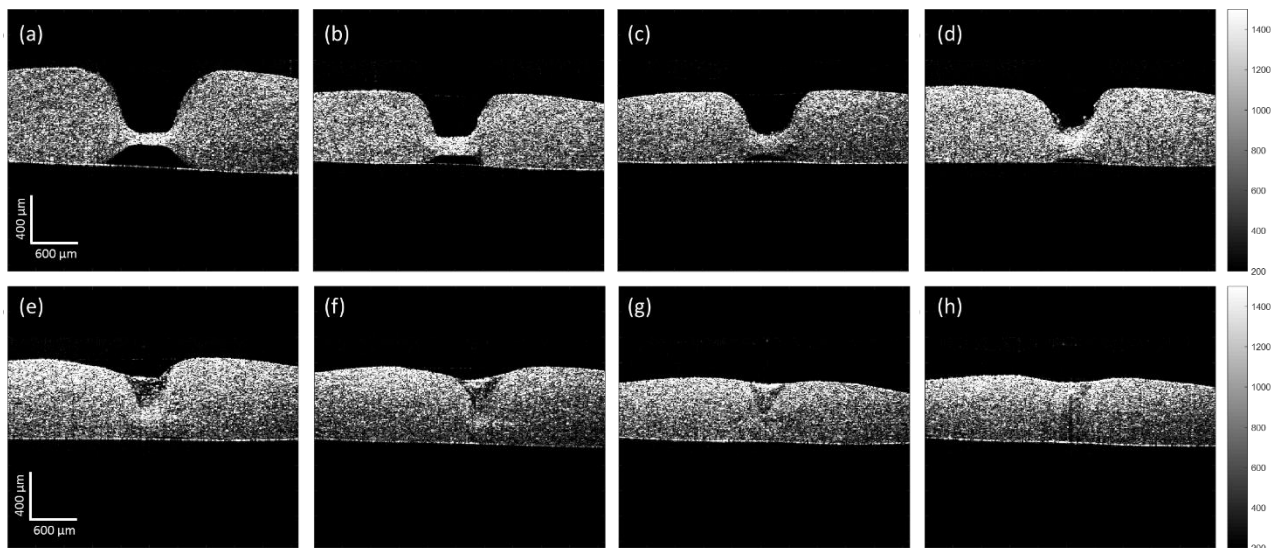


Figure 4. Cross-sectional OCT images of corneal wound healing process at (a) Day 0, (b) Day 1, (c) Day 2, (d) Day 4, (e) Day 7, (f) Day 9, (g) Day 11 and (h) Day 14.

It is noted that during the wound healing process the overall thickness of the corneal model is getting thinner. As shown in Table 1, the corneal thickness contracted in the first 24 hours, but there was only little fluctuation in the next 8 days. It has an original overall thickness of 857 μm and decreased 32.56% after two weeks. Little amount of new tissue pieces started to appear after 4 days as shown in Figure 4(d). These new tissue is more obvious in a week time as illustrated in Figure 4(e) with loose structure across the lesion area. Since then, the new tissue became increasingly denser and the lesion fully closed after two weeks. The lesion depth was measured at the centre of the punched hole to the top surface of the cornea model. In the case of new tissue generated, the lesion depth was the distance between the top surface of new tissue and the top surface of the corneal model. Table 1 presents that the lesion depth decreases from 560 μm to 50 μm during 14 days. The ratio of lesion depth to the overall corneal thickness also indicates the successful healing procedure of the mimicked corneal wound.

Table 1. Parameters of corneal wound healing with a punched lesion.

	D0	D1	D2	D4	D7	D9	D11	D14
Corneal thickness (μm)	857	635	621	659	700	671	536	578
Lesion depth (μm)	560	383	383	342	153	101	36	50
Ratio (%)	65.35%	60.28%	61.59%	51.88%	21.86%	15.10%	6.72%	8.56%

3.3 3D visualization of corneal wound healing

Three-dimensional (3D) images of corneal wound healing process are illustrated in Figure 5 of the corneal model with a 1-mm punched lesion. OCT images in Figure 5 provide a 3D visualization of the change of lesion size and depth during the corneal healing process. It is noted that there is still a shallow hole at the top surface of the newly generated tissue even after two weeks' culture.

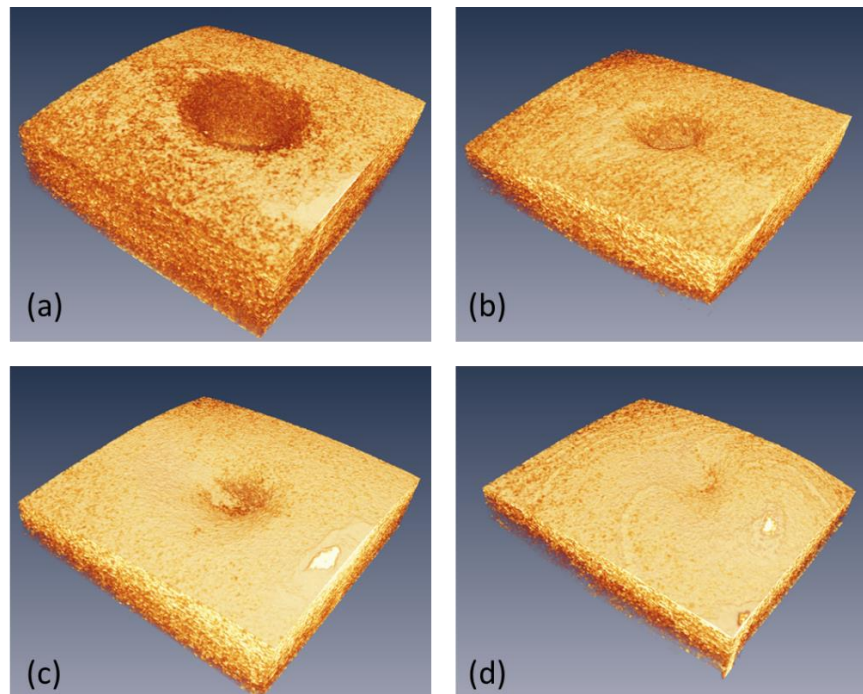


Figure 5. 3D visualization of corneal wound healing process at (a) Day 0, (b) Day 4, (c) Day 7 and (d) Day 14. Image size of sampling area is 3.5 mm by 3 mm horizontally whilst the thickness varies.

3.4 Mechanical evaluation of corneal wound

A corneal model with a 1.5-mm punched lesion was illustrated in Figure 6. After adding vibration to the sample holder, the tissue displacement was detected by the OCT light. The details of the principle was verified in the previous study¹⁴. Typically, Figure 6(a) denotes the sinusoid vibration signal generated versus time from a red point in the corneal model in Figure 6 (c). After Fourier transform (FFT) of the vibration signal, a peak amplitude at the modulated frequency is related to the displacement as shown in Figure 6(b) in order to calculate the strain value. Therefore, the elastogram (Figure 6 (d)) can be generated with the reciprocal of the strain value. The generated elastogram is colour coded with red to be stiff and blue to be soft. After 7 days' culturing, newly generated tissue is softer than the surrounding normal area in the corneal model. It is observed that the new tissue is not intact and the mechanical strength is yet recovered after a week.

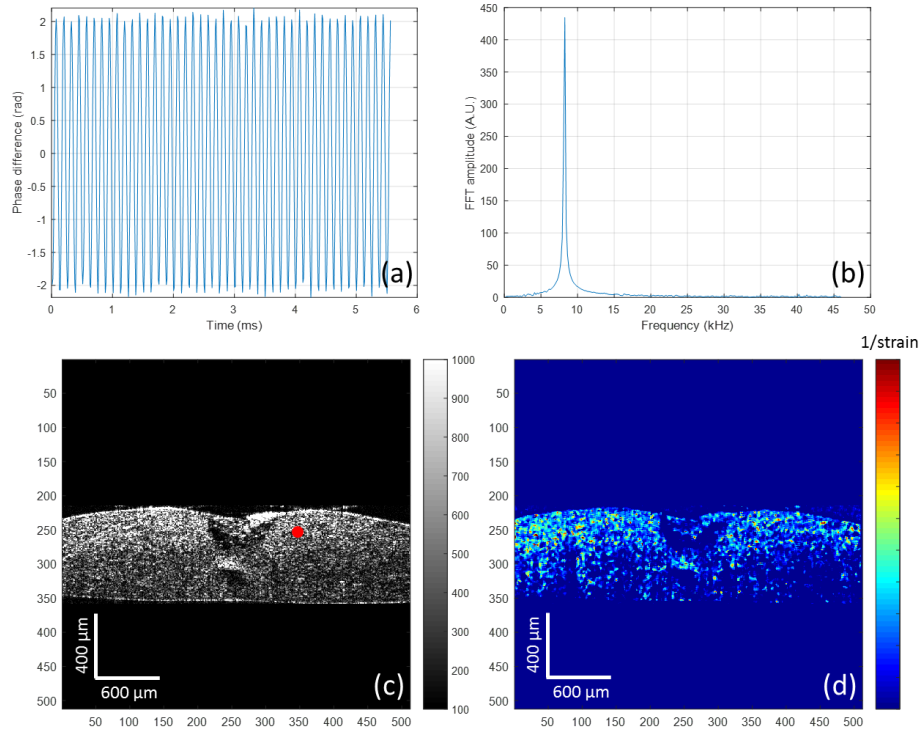


Figure 6. Mechanical evaluation of a corneal model with a 1.5-mm punched lesion after a week. (a) Vibration signal along time of a typical point in (c), (b) FFT amplitude of the phase difference as a function of frequency, (c) the structure OCT cross-sectional image and (d) the elastogram of the corneal wound.

4. DISCUSSION AND CONCLUSION

In this preliminary study, an *in vitro* human 3D corneal model was successfully established and corneal ulcer was mimicked with various size and depth. It was observed that the human miniature corneas contracted during culturing time due to the nature of collagen reorganization and interaction with fibroblasts. In this study, the horizontal contraction was controlled with a cellulose ring and the longitudinal contraction was monitored with precisely controlled timeline. The results present the reduction of both the corneal thickness and the lesion depth, as well as the ratio of the lesion depth to the overall corneal thickness. It suggests that although the contraction of the corneal equivalents can change lesion shape, the lesion healing process might play a more important role. Moreover, the newly generated tissue observed with OCT/OCE significantly manifests the wound healing process was functioning. It was also noted that the mechanical strength of newly generated tissue is weaker than the surrounding tissue.

In summary, a real-time and non-contact technique was introduced with OCT/OCE images indicating the tissue regeneration. Such 2D/3D visualization of the corneal wound and the depth information could be of great clinical impact to understand the biomechanics of the cornea healing process and the therapeutic effectiveness of regenerative medicine.

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